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Use of fluorescent *in-situ* hybridisation to investigate possible involvement of *Clostridium* spp. during softening and fermentation of cassava

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Abstract. This study was designed to investigate the possible role of *Clostridium* spp. in softening of cassava, using a novel molecular method "fluorescent in-situ hybridisation" (FISH). The technique can be applied directly to fermented cassava, and is used in parallel with conventional cultural isolation techniques. The use of FISH has been proposed to validate the results of cultural techniques and to determine the presence of components of the microflora that were not previously recovered. Peeled and unpeeled cassava pieces were incubated at 40°C, 37°C, 30°C and 25°C for 5 days. Compiled results of current and previous *in vitro* model cassava fermentations, demonstrated large variations in the extent of softening during fermentation. In the most recent study, however, the fermentation did lead to cassava softening. Although low numbers of presumptive *Clostridium* spp. were recovered using conventional cultural methodology, results obtained using the FISH technique failed to demonstrate the presence of this bacterium. It was concluded that, while there is some evidence for the presence of *Clostridium* spp. in cassava fermentations, further work on the methodology of FISH assays is required. Possible reasons for the failure of the technique to detect Clostridia are discussed.

Introduction

Cassava (*Manihot esculenta* Crantz) is an important starchy staple food consumed mostly in the tropics. It is processed into various local products, thus providing a basic daily source of dietary energy. It is, however, associated with major nutritional limitations, being low in protein and micronutrients. It is often utilised in sub-Saharan Africa as the main diet of infants, and young children during the weaning and post weaning stage. Long term feeding, however, results in severe malnutrition due to a combination of protein and various micronutrient deficiencies (Mosha *et al*., 2000). Cassava contains toxic cyanogenic glucoside compounds that can produce toxic levels of hydrogen cyanide in the range from 10 - 450 mg/kg of fresh root depending on variety (Tokuibiye *et al*., 1996). Regular consumption of inadequately processed cassava can lead to chronic toxicity (Holzapfel, 2002). Various traditional processes exist for detoxicification to improve cassava safety and digestibility before consumption. Fermentation reduces levels of cyanogens due to degradation by linamarinase (a-glucosidase) produced by lactic acid bacteria (Lei *et al*., 1999). A reduction in cyanogen level results from soaking and retting and culinary activities, such as slicing, cutting and grating, that cause cell disruption (Gidanis *et al*., 1993).

Cassava can be classified as sweet or bitter depending on the cyanogenic glucoside levels. Bitter cultivars are generally used as raw material for starch production, except in Africa, where they are consumed by humans. Variations in cyanogenic glucoside levels in cassava due to growing conditions, soil type,

moisture, temperature and age of the plant are superimposed on variations between cultivars. Cassava root has a major post-harvest constraint in being highly perishable, the poor keeping quality being caused by a number of factors, including physiological and microbiological deterioration (Borges *et al*., 2002). Physiologic deterioration leads to vascular discoloration (streaking). Microbiological deterioration also occurs and is usually a consequence of tuber damage during harvesting and handling operations. Therefore, cassava must be processed into a storable form soon after harvest, if losses are to be minimised.

Traditional "wet" process for cassava. The submerged traditional cassava fermentation is spontaneous, uncontrolled and is associated with a complex indigenous microflora (Okafor *et al*., 1984). It involves a lactic fermentation characterised by acidification, preceded by a retting stage, during which softening of the cassava may occur. It has been proposed that during submerged fermentation, pectin-degrading enzymes disrupt the cell membrane and alter its integrity. This process enhances removal of toxins through leaching into water during soaking (Westby *et al*., 1997; Okafor *et al*., 1984). Experiments with small-scale fermentations have shown a lack of reproducibility in the softening process. Observations were based on results of several repeat trials using both peeled and unpeeled cassava. These demonstrated a wide and unpredictable variability of cassava softening from batch to batch. At present, there is no rationale, which can fully explain such variations in the softening process; thus the underlying mechanism remains obscure and hypothetical. It is anticipated that full elucidation of the cassava softening mechanism will be a component key of strategies enabling optimisation of cassava fermentation and quality of end product. A number of studies have suggested that both bacterial enzymes and the endogenous enzymes of the cassava are implicated (Okafor *et al*., 1984, Oyewole, 1992, Amoa-Awua, 1999, 1995; Holzapfel, 2002). Despite this, the origin of pectinolytic enzymes in cassava fermentations remains obscure. No known member of the dominant lactic acid bacteria produces these enzymes and it is unlikely that these bacteria play a role in softening. Amongst the Enterobacteriaceae, which can also reach significant numbers in the earlier stages of fermentation, *Erwinia* spp. are well known for production of pectinolytic enzymes and associated cell disruption. There is no evidence, however, for a role for *Erwinia*. It has further been postulated that *Clostridium* spp. may be responsible for softening, but this also has not been substantiated. There is, however, supportive circumstantial evidence. This includes a strong odour of butyric acid, a fermentation end-product of some clostridia, when softening occurs but not in its absence.

The genus *Clostridium*. The genus *Clostridium* comprises Gram-positive, anaerobic endospore forming, rods. Although all members of the genus are obligate anaerobes, the degree of aero tolerance varies widely. The bacterium is common in a number of environments, including soil, marine and fresh water, and in human and animal intestinal tracts. Carbohydrate fermentation is a common property of clostridia, but alternative energy-yielding metabolic pathways exist in the group, including amino acid fermentation. *Clostridium butyricum* and a number of other clostridia ("butyric acid clostridia") are able to ferment soluble carbohydrates, starch or pectin with the formation of end products such as acetic and butyric acids, CO_2 and H_2. In many natural environments *Clostridium* is of importance in mineralisation of organic matter and is a major component of the microflora that develops during the "retting" process. Retting is a traditional process, involving the softening of plant materials immersed in water. Degradation of pectin and other materials that give the plant cells their integrity is an important part of the retting process.

Cultural vs non-cultural methods in the study of fermentations. Until relatively recently, investigations of the microorganisms involved in fermentations were dependent on cultural methods, usually based on colony counting techniques. A major, and well-recognised, problem with cultural techniques is that only a relatively small proportion of the bacteria present is recovered. This can be a result of stress, and entry of bacteria into a viable, non-recoverable (VNR) state has received considerable attention when studying the behaviour of foodborne pathogenic microorganisms. In many cases, however, failure to recover microorganisms is a consequence of limitations in the colony counting procedure. Colonies may fail to develop for a number of technical reasons, including unsuitable composition of media, incorrect incubation conditions, or competition from other microorganisms that are better able to develop colonies on the agar surface. In some cases, it is possible to enhance recovery by modifying the media used and apparently minor changes in incubation conditions can also result in major improvements. Despite this, in many environments, a significant proportion of the microorganisms present remains unrecoverable by conventional cultural techniques. Descriptions of processes mediated by microorganisms, including fermentations, have tended, therefore, to give undue weight to those components of the microflora that are able to grow most rapidly on the media used. The development of culture-independent molecular techniques that are capable of determining the composition of complex ecosystems has greatly expanded the knowledge of many ecosystems. In habitats as diverse as the human gut and sub-surface sediments, for example, it is now recognised that the great majority of species cannot be recovered by cultural means. In the case of food fermentations, the assumption is often made that population structure is simple and dominated by one, or a small number, of microorganisms. While this assumption may be correct in the case of dairy or brewery fermentations where starter cultures are added to a heat-treated substrate, molecular methods have revealed much greater diversity in fermentations, including plant material, than previously thought to exist (Ampe *et al*. 2001). Although molecular methods offer a powerful means of determining diversity and population structure in fermentations, use until relatively recently has been limited. This results from unfamiliarity with the techniques and cost and availability of materials such as primers. Newer techniques, such as fluorescent in-situ hybridisation (FISH), however, are relatively simple and allow microorganisms present in virtually any ecosystem, including fermentations, to be detected and identified at various levels of discrimination up to genus. Other molecular techniques have been developed, including denaturing gradient gel electrophoresis. This technique is discriminatory to species level, but requires a significantly higher level of manipulations than FISH.

FISH is a molecular tool based on DNA sequencing that enables simultaneous direct visualisation and identification of individual microorganisms. The technique provides a powerful means of detecting and characterising microorganisms such as *Clostridium* spp. that can be difficult to isolate using conventional cultural techniques (Moter and Gobel, 2000; Rycroft *et al*. 2001). The underlying basis for FISH is that any isolated DNA sequence or gene can be mapped to a specific bacterial chromosome. This enables labelled cells, or chromosomes, to be identified according to the nucleic acids contained within them. Fluorescently labelled pieces of nucleic acid (probes) of approximately 20 nucleotides in length are used. The probes are incubated in the presence of cells under appropriate conditions of temperature and ionic strength to permit specific hybridisation of probe to target nucleic acid. Cell types that contain complementary RNA sequences become labelled by the binding of the fluorescent probe *in-situ*. Labelled cells are then

visualised by fluorescent microscopy or flow cytometry.

Although FISH is a relatively straightforward technique, there can be difficulties in its use. The technique, for example, is dependent on availability of suitable primers, and the concentration of genetic materials of the specimen. There are also potential technical problems. Detection by fluorescent microscopy, for example, is relatively insensitive. Further, examination of a large number of samples is tedious and can be error-prone. Flow cytometry is automated and capable of high throughput, but equipment is expensive and considerable effort can be required to establish a working protocol. Despite the problems, FISH and other molecular techniques are highly effective tools for determining the microorganisms present in a wide range of ecosystems. Molecular techniques do not, however, replace the need for cultural methods, since at some stage full studies of the microorganisms using pure cultures is required. The most effective picture of fermentation, or any ecosystem, is usually, therefore, obtained by a combination of conventional cultural and molecular methods. This approach is being adopted in the current project, where the use of FISH is being investigated to determine the presence of Clostridia and any differences between cassava fermentations that exhibit softening and those that do not.

Aim of study. To investigate the possible role of *Clostridium* spp. in softening of cassava, using a non-cultural technique, fluorescent in-situ hybridisation in conjunction with classic cultural methodology. It is meant to provide data to support existing circumstantial evidence that *Clostridium* spp. is present in significant quantities during the cassava fermentation.

Materials and Methods

Model cassava fermentations. Cassava tubers were purchased in local shops in North London. These tubers were mostly of the sweet variety and were imported mainly from Ghana and Nigeria. Their age was not known. The tubers were all characterised by a waxy, shiny skin mainly devoid of soil. It was probable that the tubers had been pre-treated to retard the post harvest deterioration, and thus extend the shelf life. There was usually, however, indications of the onset of spoilage. Peeled and unpeeled cassava tubers were cut into cylindrical pieces of 6 cm diameter and 1 cm width. A total of 150 g of each type of cassava was added to 300 ml of distilled water in lidded jars. The jars were tightly closed and incubated in air at 40°C, 37°C, 30°C and 25°C respectively for 5 days. Sampled were taken immediately after preparation and after 1, 3 and 5 days for examination by conventional cultural techniques and by FISH.

Conventional cultural microbiology. Three media were used for detection and enumeration of *Clostridium* spp. These were reinforced clostridial agar (RCA), Columbia blood agar base containing horse blood and supplemented with polymyxin B (CBP) or neomycin (CBN). In the earlier part of the work, anaerobic conditions were obtained by use of gas generators (Oxoid, UK) in anaerobic jars and media were not prereduced. Subsequently, media and diluent was prereduced and isolation plates incubated in an anaerobic workstation (Don Whitley Scientific, UK) at 37°C for 48 hours. Isolates were presumptively identified as clostridia on the basis of Gram-stain and cell morphology and on inability to grow during incubation in air.

Lactic acid bacteria (LAB) were enumerated on de Mann, Rogosa & Sharpe agar (MRS; Oxoid UK)) incubated anaerobically at 37°C for 5 days. Isolates were presumptively identified and assigned to species on the basis of Gram-stain and cell morphology and a negative catalase reaction. pH value was determined electronically.

Application of fluorescent *in-situ* hybridisation

Step 1: Fixation. Fermented cassava samples (10 g/each) were removed from the ferment, homogenised and centrifuged. The supernatants were treated with denaturing agents (pH 7.2) and stored at 4°C overnight to fix bacterial cells. Fixation is required to maximise penetration and retention of the probe, to remove possible interfering compounds and to protect the RNA from degradation by endogenous ribonucleases. The fixed cells were then washed in buffer solutions of pH 7.0.

Step 2: Hybridisation. The fixed bacterial cells (135µl) were mixed with fluorescent probe (50 ng/µl^{-1}: 15µl) and incubated at 55°C overnight in aqueous salt solutions. A commercial genus specific 16 rRNA targeted oligonucleotide probes labelled with the fluorescent dye Cy3 was used to detect *Clostridium* spp. Cy3 is used as dye to enhance brightness. The hybridised cells were subjected to washes in buffer solutions of appropriate strength.

Step 3: Post hybridisation. 6-diamidino-2-phenylindole (DAPI) solution (500 ng/ µl^{-1}) was added to hybridised cells as blue fluorescent counterstain, and placed in a hybridisation oven for 30 minutes at 55°C. The unbound probe was removed by washing in buffer.

Step 5: Mounting and visualization. The hybridised cells were filtered (0.2 um pore membrane) and the filters were placed on glass slides and SlowFadeÒ (Molecular Probes Inc, The Netherlands) was added to each filter. Cells were visualised using a Nikon Eclipse E400 microscope (Nikon, UK) fitted with filters for the DAPI stain (excited at 359 nm and emitting at 461 nm) and the Cy3 dye (excited at 550 nm and emitting at 565 nm).

Results and Discussion

Following the analysis of the outcome of fermentations, three main observations were made concerning softening.

- Softening was always accompanied by a strong odour, characteristic of butyric acid. The precise patterns led us to propose that this odour is produced by clostridia and that there may be a causal relationship between these bacteria and softening. This observation may agree with previous reports
- The degree of softening was variable under the same fermentation conditions. This was independent of size of cassava pieces. Various sizes of cassava were investigated, and the outcome was that in some cases the entire pieces softened. In other batches, only the outer part of cassava pieces softened, while the inner part was still hard. Occasionally one batch would contain a mixture of fully and partially softened cassava pieces. This strongly disagreed with previous findings with suggestion of influence of size (Okafor *et al*., 1984).
- In every fermentation medium, pH was low due to acidification by lactic acid bacteria. Therefore, the low pH value was not related to softening. There was no consistent relationship between softening and specific parameters including temperature and time of fermentation or final pH. Although there was some inconsistency in results obtained from comparisons of peeled and unpeeled cassava, it was observed that softening was more common when cassava was peeled. There have been other indications that the skin of cassava contains compounds that can alter the course of the fermentation. This phenomenon has not yet been investigated systematically.

After an initial lag of 3 to 4 hours, growth of lactic acid bacteria was rapid and high numbers (~ 10^8 cfu/g) were present after 24 hours, increasing only slightly as the fermentation continued. Softening was independent of both acidification rate and final pH and, for this reason, it is considered that lactic acid bacterial are not responsible for cassava softening.

Presumptive *Clostridium* spp. were detected on Columbia blood agar, supplemented with polymyxin B, in numbers up to 5 x 10^5 cfu/g when an anaerobic workstation was used and full precautions were taken to protect cells from oxygen. Recovery using anaerobic jars was markedly lower. Recovery was also lower on Columbia blood agar supplemented with neomycin, while reinforced clostridial medium was ineffective due to overgrowth by facultative anaerobes. Higher numbers were isolated when softening was extensive and the characteristic odour was present. At present, insufficient results are available to imply a causal relationship. Levels of clostridia in the order of 5 x 10^5 cfu/g would not, however, be expected to be sufficient to cause softening. Clostridia can, however, be difficult to recover, partly due to variation in spore germination. The increase in recovery when using fully anaerobic conditions is considerable, suggesting that the dominant clostridia are poorly adapted to growth on artificial media. This possibility requires further investigation.

It is proposed to extend the incubation time from 48 to 96 hours to permit full adaptation to growth in artificial media.Initial results of the FISH technique failed to demonstrate the presence of *Clostridium* spp. This may suggest that the colony counts are accurate, since the limit of detection using fluorescent microscopy is ~10^5 cells. It is also possible that the primers used are not suitable, or that signal strength is weak, due to low cellular ribosome content, a common problem with environmental samples (Amann, 2000). This can be a particular problem with unusual species. There have also been unsubstantiated reports of food components interfering with binding. FISH is also highly dependent on technique (Moter & Gobel, 2000; Amann, 2000), and it is possible that improved results can be obtained through relatively minor modifications to the methodology.

Implications

It has been reported that the final product of the traditional cassava fermentation process is subject to quality variability (Sanni, 1993). A full understanding of the mechanism of cassava softening during fermentation, particularly at industrial scale, would provide opportunities for optimasing the process. This would consequently increase the yield, improve the quality of the end - product and ensure a better market for it.

References

Amann, R. 2000. Methodological Aspects of Fluorescence *In Situ* Hybridisation, Bioscience and Microflora 19:85-91.

Amoa-Awua, W.K.A. and Jakobsen, M. 1995. The role of *Bacillus* species in the fermentation of cassava. Journal of Applied Bacteriology 79:250-256.

Ampe, F., Sirvent, A. and Zakhia, N. 2001 Dynamics of the microbial community responsible for traditional cassava fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization, International Journal of Food Microbiology 11:45-54.

Bockett, G. 1998. *Safe methods of using better cassava varieties: an extension guide*. Publication funded through the Crop Post-Harvest Programme of the United Kingdom's Department for International Development, London: UK.

Borges, M.F., Fukuda, W.M.G. and Rossetti, A.G. 2002. Evaluation of cassava varieties for human consumption. Pesquisa Agropecuaria Brasileira 37 (11):1559-1565.

Brennan, J.G., King, R.D. and Muzanila, Y.C. 2000. Residual cyanogens, chemical

composition and aflatoxins in cassava flour from Tanzanian villages. Food Chemistry 70: 45-49.

Holzapfel, W.H. 2002 Appropriate starter culture technologies for small-scale fermentation in developing countries. International Journal of Food Microbiology 75, 197-212IITA: http://www.iita.org/crop/cassava.htm ,10/05/03.

Gidamis, A.B., O'Brien, G.M. and Poulter, N.H. 1993. Cassava detoxification of traditional Tanzania cassava foods. International Journal of Food Science and Technology, 28: 211-218.

Lei, V., Amoa-Awua, WKA. and Brimer, L. 1999 Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from spontaneous cassava fermentation and other microorganisms. International Journal of Food Microbiology 53: 169-184.

Mosha, T.C.E, Laswai, H.S. and Tetens, I. 2000 Nutritional composition and micronutrient status of home made and commercial weaning foods consumed in Tanzania. Plant Foods and Human Nutrition 55:185-205.

Motarjemi, Y. 2002. Impact of small-scale fermentation technology on food safety in developing countries. International Journal of Food Microbiology 75: 213-229.

Moter, A. and Gobel, U.B. 2000). Fluorescence *in situ* hybridisation (FISH) for direct visualization of microorganisms. http://nri.org/reseach/rootcrops.htm, 10/05/03.

Okafor, N., Ijioma, B. and Oyolu, C. 1984. Studies on the microbiology of cassava retting for foo-foo production. Journal of Applied Bacteriology 56: 1-13.

Oyewole, OB. and Ondunfa, SA. 1992 Extracellular enzyme-activities during cassava fermentation for fufu production. World Journal of Microbiology and Biotechnology 8: 71-72.

Rycroft, C.E, Jones, M.R., Gibson, G.R. and Rastall, R.A 2001 Fermentation properties of gentio-oligosaccharides. Letters in Applied Microbiology 32:156-161.

Sanni, A.I. 1993 The need for process optimisation of African fermented foods and beverages. International Journal of Food Microbiology 18:85-95.

Tokuibiye, G., Sokari, P. and Karibo, S. 1996. Short duration processing for high quality gari production. Journal of the Science of Food and Agriculture 70: 427-432.

Westby, A., Reilly, A. and Bainbridge, Z. 1997. Review of the effect of fermentation on naturally occurring toxins. Food Control 8:329-339.